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Developmental and functional heterogeneity of monocytes

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Running Title: Monocyte heterogeneity

Summary

The past decade brought fresh insight into monocyte development and function, with the aid of innovative experimental models such as fate-mapping and single-cell analysis, which redefined the monocyte field. Monocytes in both rodents and humans are now known to consist of multiple subsets generated through distinct developmental pathways with diverse functional specializations. Monocyte fates under homeostatic conditions include the accumulation in peripheral reservoirs, the conversion into patrolling monocytes and the engraftment into certain resident macrophage pools. Furthermore, under pathological conditions monocytes can acquire effector functions such as inflammatory or antigen presentation activities, but can also develop regulatory properties essential for tissue repair. Importantly, monocytes recruited during inflammation are often functionally distinct from resident macrophages or conventional dendritic cells. Here we propose a historical overview of the monocyte field and highlight how novel concepts in monocyte heterogeneity, emergency monopoiesis and trained immunity are bringing exciting new perspectives to monocyte research.

Monocytes: not only the immature siblings of macrophages and dendritic cells.

Monocytes make up one component of the "mononuclear phagocyte system" (MPS), which they share with terminally differentiated tissue macrophages and dendritic cells (DC) (Guilliams et al., 2014). Monocytes are defined as circulating blood cells that constitute ~10% of peripheral leukocytes in humans and ~4% of peripheral leukocytes in mice. Blood monocytes are thought to develop in the adult bone marrow (BM) from a dividing common myeloid progenitor (CMP) that is shared with erythrocytes, platelets, DC and granulocytes. Following their generation in the BM, monocytes are subsequently released into the peripheral circulation (van Furth et al., 1979). Historically, monocytes were believed to represent a bridge linking BM precursors via a circulating stage with terminally differentiated macrophages and DC in the tissues. Today we appreciate that in most tissues the majority of resident macrophages - but not all - have an embryonic origin and bypass a monocyte stage in the adult, while DC derive from distinct BM precursors and not from monocytes (Ginhoux and Guilliams, 2016; Mildner and Jung, 2014). Therefore, during the last decade, monocytes have freed themselves from their exclusive image as simply an immature sibling of macrophages or DC. Indeed, recruited monocytes during inflammation give rise to monocyte-derived cells that are functionally distinct from the resident macrophages and DC. Moreover, circulating monocytes can consist of distinct subsets that already possess functional properties before reaching the inflamed tissues. As such, the leading characteristic that distinguishes monocytes from other members of the MPS is that they are poised to be rapidly mobilized in large numbers to sites of infection or injury throughout the body, where they serve as particularly plastic "emergency squad" to provide pro-inflammatory or resolving activities. These diverse effector functions can be further shaped in part by micro-environmental cues and spatial context.

Monocytes lost in history

The earliest accounts of monocytes are closely related with the introduction of the microscope in the mid-nineteenth century (Fig. 1). Whilst still a medical student, Paul Ehrlich was developing leukocyte cytological stains. By using aniline dyes in combination with neutral dyes and the nuclear morphology of leukocytes, he was able to compartmentalize white blood cells into mononucleated leukocytes, some of which he termed large mononuclears with kidney shaped nuclei or so called Übergangszelle (*transitional cells*; now known as monocytes) while others he described as polymorphonuclear cells with neutrophilic (neutrophils), acidophilic (eosinophils) or basophilic granules (basophils) (Ehrlich, 1880). In 1908, Paul Ehrlich shared the Nobel Prize in Physiology or Medicine with Ilya Metchnikoff "in recognition of their work on immunity". Ilya Metchnikoff was at this time establishing his theory of phagocytosis, giving rise to the concept of cellular innate immunity (Gordon, 2008; Metchnikoff, 1887; Metchnikoff, 1892; Yona and Gordon, 2015).

The term 'monocyte' was – to the best of our knowledge – introduced by the German physician Artur Pappenheim in 1910 (Pappenheim and Ferrata, 1910). Pappenheims goal at this time was to unify a

wide variety of previously described cell types into one major subgroup – the large monocyte[1]. Over the subsequent years, the definition of monocytes became more and more stringent and was exclusively applied to Ehrlich's *transitional cells* (Forkner, 1930; Klein, 1914; Naegeli, 1923; Sabin et al., 1925). Attention soon turned to the origin of monocytes and gave rise to a multitude publications and debates (Forkner, 1930; Naegeli, 1923; Pappenheim, 1919).

In 1914, Awrorow and Timofejewskij conducted the first *in vitro* cell culture experiment of human leukocytes isolated from peripheral blood from a leukemic patient and concluded that lymphocytes are the progenitors from which macrophages arise (Awrorow and Timofejewskij, 1914). Awrorow also observed that these *in vitro* derived macrophages do not form a homogenous population, but instead can vary in morphology and function, depending on the culture condition and thereby foreseeing the plasticity of monocytes (Awrorow and Timofejewskij, 1914). The first *in vivo* study to observe the migratory behaviour of mammalian blood monocytes and differentiating into macrophages during an acute pathological insult was performed by Ebert and Florey (1939), using the rabbit ear chamber (Ebert and Florey, 1939). This rapid and substantial influx of monocyte-derived inflammatory macrophages at the site of inflammation led to the conclusion that the majority of inflammatory macrophages are derived from circulating monocytes rather than descendants of local resident macrophage proliferation (Leder, 1967).

By the late 1960's a wealth of knowledge had accumulated in relation to the monocyte and macrophage, leading Ralph van Furth, James Hirsch, Zanvil Cohn and colleagues to introduce the term MPS (van Furth et al., 1972), established on "similarities in the morphology, function, origin and kinetics of the phagocytes" (van Furth et al., 1972). They concluded that pro-monocytes in the BM act as the precursor of circulating monocytes, which can differentiate into macrophages in the tissues. It is important to recall that at this time, macrophages were described as either free or fixed. These fixed macrophages were defined, as "probably of monocyte origin, but definitive proof has not been obtained…the morphology and functional behaviour…justify their inclusion in the mononuclear phagocyte system". The concept that fixed macrophages would be of monocyte origin was then incorporated as textbook knowledge and the cautionary note forgotten. Subsequent technological advances permitted the experimenter to examine the relationship and turnover of monocytes and macrophages. Tissue-resident macrophages for instance are characterized by longevity (Bouwens et al., 1986; Melnicoff et al., 1988). Furthermore, ^{89}Sr-induced monocytopenia in animals did not affect normal tissue-resident macrophage compartments and macrophage cell numbers (Naito and Takahashi, 1991; Oghiso et al., 1988; Sawyer et al., 1982; Yamada et al., 1990). Interestingly, histological approaches demonstrated the first appearance of tissue colonization by fetal macrophages during embryonic development occurs before the establishment of definitive haematopoiesis (Mizoguchi et

[1] „Solange diese Frage noch nicht geklärt und ihre Histiogenese, Natur und Stellung strittig ist, empfiehlt es sich, diese Zellklasse als ‚große Monozyten' zu bezeichnen." – As long as the question is not solved and their histogenesis, nature and position controversial remains, it is recommended to name this class of cells as 'large monocytes'

al., 1992; Naito and Takahashi, 1991; Sorokin and Hoyt, 1992) These results suggested that at least some tissue-resident macrophage populations are independent of monocyte influx and show potential self-renewal capacity, thereby leaving the question open: what exactly are the functions of monocytes?

Monocyte subsets

Ehrlich's *transitional cell* was described as a homogenous cell population with the unifying histological feature of a centrally located kidney shaped nucleus. However, the advent of flow cytometry has had an unrivalled impact on immunology by enabling the assessment of cellular heterogeneity. This has facilitated the identification and stratification of a number of monocyte subsets in a variety of species (Ziegler-Heitbrock, 2014), including humans (Passlick et al., 1989), mice (Geissmann et al., 2003), rats (Ahuja et al., 1995; Barnett-Vanes et al., 2016), pigs (Sanchez et al., 1999) and monkeys (Kim et al., 2010). Discrete populations of monocytes were first identified by morphology and the differential expression of the antigenic markers CD14 and CD16 in humans (Passlick et al., 1989; Wong et al., 2011) and represented a major milestone in monocyte biology. The combination of CD14 and CD16 on human HLA-DR^{+} cells enabled the classification of three principal human monocyte subsets: CD14^{+} CD16^{-} monocytes, also referred to as 'classical' monocytes, make up 80-90% of the monocyte pool with the remaining 10-20% shared by CD14^{+} CD16^{+} intermediate and CD14^{Low} CD16^{+} 'non-classical' monocytes. A major breakthrough in the characterisation of mouse monocytes arose with the generation of the CX$_3$CR1^{GFP} *knock-in* mouse (Jung et al., 2000), which led to the identification of two distinct CD11b^{+} CD115^{+} monocyte subsets with different biological functions (Geissmann et al., 2003). In mice 'classical' monocytes are characterized by the surface marker combination Ly6C^{Hi} CX$_3$CR1^{int} CCR2^{+} CD62L^{+} CD43^{Low} (previously termed inflammatory monocytes), while 'non-classical' monocytes are defined as Ly6C^{Low} CX$_3$CR1^{Hi} CCR2^{Low} CD62L^{-} CD43^{+} (Geissmann et al., 2003; Jakubzick et al., 2013; Jung et al., 2000; Palframan et al., 2001). Transcriptional profiling comparison between mouse and human monocytes correlated Ly6C^{Hi} monocytes with human 'classical' monocytes and Ly6C^{Low} monocytes with their 'non-classical' CD14^{Low} CD16^{+} human monocyte equivalent, even though certain gene expression and surface marker differences exist. First, human monocytes can be identified by their HLA-DR expression, while only certain Ly6C$^{Hi\text{-}to\text{-}Int}$ monocyte subsets express MHCII in mice. Furthermore, mouse monocytes, but not their human equivalent, are characterized by the existence of a peroxisome proliferator-activated receptor γ (PPARγ) signature and exhibit differences in genes involved in phagocytic activity (Cros et al., 2010; Ingersoll et al., 2010).

The ratio between classical and non-classical monocytes varies between species, as in humans the ratio is in favour of classical monocytes while in mouse both subsets are represented equally. However, as we will discuss below, recent unbiased single-cell RNA sequencing studies have broadened the heterogeneity within the intermediate population with the identification of several distinct subsets in

humans (Villani et al., 2017) as well as in mice (Mildner et al., 2017). Their function and ontogeny is currently unclear.

Development of classical monocytes

Over a century ago Alexander Maximow postulated that haematopoiesis of distinct blood lineages would be a highly ordered process arising from common precursors that become progressively more restricted ultimately producing diverse populations of blood cells (Maximow, 1907). This tree-like hierarchy is modelled on the assumption of a canonical development, where a set of oligo-, bi- and unipotent progenitor cells become progressively more restricted and give rise through branching decisions to all forms of circulating blood cells (Patel and Yona, 2018)(Fig. 2a). In this model, a fraction of active haematopoietic stem cells (HSC (Busch et al., 2015)) have the ability to self-renew to generate a heterogeneous population of multipotent progenitors (MPP; (Pietras et al., 2015)) that further differentiate into two separate lineages, becoming either common myeloid progenitors (CMP; (Akashi et al., 2000)) or common lymphoid progenitors (CLP; (Kondo et al., 1997)). The CLP give rise to all lymphoid blood cells including T, B and natural killer cells but lack the potential to differentiate into the myeloerythroid lineage, while CMP have lost their lymphoid lineage capacity, but are instead able to differentiate into megakaryocyte/erythrocyte progenitor (MEP) and granulocyte/macrophage progenitors (GMP) (Akashi et al., 2000). A monocyte/DC progenitor (MDP) was subsequently identified within, and probably derived from, the GMP population, which was able to give rise to monocytes and DC but not neutrophils (Fogg et al., 2006; Olsson et al., 2016), a similar finding to the situation in humans (Lee et al., 2015). However, the murine MDP was also identified by fluorescent-activated cell sorting (FACS) within the CMP compartment and might directly emerge from Flt3-expressing CMP and not exclusively by GMP (Yanez et al., 2017). MDP finally make a binary decision to either divide into the dedicated common DC precursors (CDP) or into the unipotent common monocyte progenitors (cMoP). The cMoP demonstrate an exclusive development potential towards monocytes, since isolated and adoptively transferred cMoPs gave rise to $Ly6C^{Hi}$ and – in a time delayed fashion – to $Ly6C^{Low}$ monocytes (Hettinger et al., 2013). cMoP have also been identified in human BM, where they were found amongst the GMP population (Kawamura et al., 2017). However, this tree-like hierarchal development is based on the isolation of FACS-purified cell populations using a limited set of predefined surface markers, followed by *in vitro* colony formation experiments or adoptive transfer approaches. Although these assays have been valuable to determine lineage potential, the genuine cell fate under physiological conditions *in vivo* is most likely more complicated. Indeed, recent advances in single-cell RNA sequencing technology has questioned the classical branching nature of haematopoietic differentiation and show for instance that lineage commitment is already evident in supposedly oligopotent progenitors (Notta et al., 2016; Perie and Duffy, 2016) (Fig. 2b). It was also shown that unipotent progenitors can directly emerge from HSC without the necessity to develop sequentially through defined intermediate bipotent precursor stages (Naik et al., 2013; Velten et al., 2017), which can be in part mediated by direct contact of

haematopoietic stem cells and lineage determining cytokines such as CSF1 (Mossadegh-Keller et al., 2013). Unbiased single-cell RNA sequencing combined with indexed FACS-sorting of early myeloid precursor subsets identified cells with a monocytic transcriptional program within the CMP as well as GMP population (Paul et al., 2015), indicating the limitation of population analysis that is based on a few membrane markers. However, the insufficient depth of sequencing in single-cell RNA transcriptome analysis might mask other lower expressed lineage potential, which are still biological relevant and thereby lead to premature conclusions in respect to lineage commitment. Indeed, single cell liquid cultures of various precursor stages in methylcellulose colony-formation assays to measure the clonal outcome showed the existence of multipotent precursors cells (Tusi et al., 2018), even though only a limited set of cytokines were used and particular cellular-derived niche signals are absent in this experimental setup. Therefore it is possible that precursor populations consist of a mixture of multi- or at least bi-potent as well as lineage pre-committed precursor cells (indicated in Fig. 2b by grey and lightly coloured cells, respectively).

The commitment towards the monocyte lineage is maintained by a distinct group of haematopoietic growth and transcription factors (Fig. 2a). Monocyte generation involves the transcription factors SPI1 (encoding PU.1), IRF8, GATA2 and KLF4, since deletion of these factors strongly perturbs monocytic precursor composition in the BM, resulting in reduced monocyte cell numbers in the periphery (Alder et al., 2008; Bigley et al., 2011; Feinberg et al., 2007; Hambleton et al., 2011; Kurotaki et al., 2013; McKercher et al., 1996; Scott et al., 2016a; Sichien et al., 2016). However, IRF8 and KLF4 seem to create a regulatory circuit, since ectopic overexpression of KLF4 in *Irf8*-deficient cells partially rescues monocyte differentiation (Kurotaki et al., 2013). Furthermore, an autosomal recessive IRF8 deficiency in humans leads to a reduced number of circulating monocytes (Hambleton et al., 2011). Also BACH2 has been shown to contribute as a negative regulator to monopoiesis and therefore *Bach2*-deficient mice have increased monocytes (Kurotaki et al., 2018). Importantly, the single-cell model (Fig. 2b) provides a different theoretical basis to study the role of transcription factors in haematopoiesis. A precursor may appear unaffected by a given mutation based on the restricted number of surface markers used for the identification of this precursor (Fig. 2a), while single-cell analysis may reveal that the mutation affects a pre-committed precursor within this pool of cells or even further upstream in the developmental tree. The MDP is for example not strongly affected by the loss of *Irf8* (Sichien et al., 2016). However, since the MDP may contain committed monocyte- and cDC1-precursors it may well be that the MDP in *Irf8*-deficient mice only consist of cDC2- committed precursors and may in fact comprise of different pool of cells as compared to wild-type MDP. This would be overlooked in the classical analysis using a restricted set of surface markers to define the MDP (Fig. 2a) but will be revealed by single-cell analysis (Fig. 2b).

Investigations into cMoP biology has uncovered that this population possess a high proliferative capacity (Chong et al., 2016; Hettinger et al., 2013; Kawamura et al., 2017) and are already characterized by Ly6C expression in mice and CD14 expression in humans, respectively (Chong et al., 2016; Hettinger et al., 2013). Further differentiation of cMoP into mature monocytes involves a pre-

monocyte stage, where these cells can be discriminated by the expression of the chemokine receptor CXCR4 (Chong et al., 2016). $CXCR4^{+}$ pre-monocytes represent a transient population with further proliferation potential and differentiate in the BM within 24 hours into $Ly6C^{Hi}$ $CXCR4^{-}$ monocytes, which is accompanied by up-regulation of the chemokine receptor CCR2 (Chong et al., 2016). Therefore, CXCR4 itself is not involved in BM egression, rather CCR2 has been shown to regulate BM exit of classical murine $Ly6C^{Hi}$ monocytes (Serbina and Pamer, 2006). It was observed that $Ly6C^{Hi}$ monocytes in CCR2-deficient mice become trapped within the BM, while the number of $Ly6C^{Hi}$ monocytes in the periphery are dramatically reduced (Serbina and Pamer, 2006). Consequently, the CCR2 chemokine ligands CCL2 and CCL7, but not CCL12, are important for $Ly6C^{Hi}$ monocyte egression from the BM since deficiencies in these genes leads to reduced levels of $Ly6C^{Hi}$ monocytes in the circulation (Tsou et al., 2007). It has been shown that under mild inflammatory conditions mesenchymal stem cells and their progeny, including CXCL12-abundant reticular cells, represent the major source of CCL2 production in the BM (Shi and Pamer, 2011), although the situation might differ under healthy conditions. If CCR2 also plays a role in monocyte egression in humans is currently unclear. Interestingly, $Ly6C^{Hi}$ monocyte egression from the BM also underlies a circadian rhythmic oscillation (Nguyen et al., 2013), which is in part CXCR4-dependent (Chong et al., 2016). CXCR4 also participates in the homing of classical and non-classical monocytes to central (BM) and peripheral (spleen) monocyte reservoirs, as the capacity of CXCR4-deficient monocytes to reach these organs is diminished (Chong et al., 2016).

Fate of classical monocytes in healthy homeostasis

Classical $Ly6C^{Hi}$ monocytes represent a transient cell population with a diverse differentiation potential. These cells, in contrast to non-classical monocytes, are equipped with a gene-expression program that enables them to migrate into various tissues under homeostatic conditions. Once classical monocytes are released into the circulation from the BM under healthy homeostasis, they remain in the circulation for approximately a day, before they either traffic to repopulate a proportion of tissue-resident macrophages in the intestine (Bain et al., 2014; Bain et al., 2013; Tamoutounour et al., 2012; Zigmond et al., 2012), dermis (Tamoutounour et al., 2013), heart (Epelman et al., 2014; Molawi et al., 2014), pancreas (Calderon et al., 2015), lung (Jakubzick et al., 2013) and testis (Mossadegh-Keller et al., 2017), or alternatively convert into non-classical monocytes (Patel et al., 2017; Yona et al., 2013). However, it should be noted that the classification of tissues based on the presence or absence of adult, short-lived monocyte-derived macrophages is becoming increasingly complex and might vary according to age, gender and genetic background. For instance, while the intestine was considered as the prototypical example of a tissue in which macrophages are short-lived and require the continuous recruitment of monocytes to sustain their macrophage pool, it has recently been shown that the intestine also harbours a $TIM4^{+}$ $CD4^{+}$ macrophage population that can self-maintain for months (Shaw et al., 2018).

Therefore, under healthy physiological conditions, extravasted Ly6C^{Hi} monocytes and their monocyte-derived descendants can be found in almost all tissues throughout the body, where they participate to a minor yet significant fraction of the local tissue-resident macrophage pool (Sawai et al., 2016). Striking exceptions of tissues in which there is little or no monocyte engraftment include the epidermis (Chorro et al., 2009; Merad et al., 2002), the central nervous system (Ajami et al., 2007; Ginhoux et al., 2010; Mildner et al., 2007) and the alveolar space of the lung (Guilliams et al., 2013; Hashimoto et al., 2013; Yona et al., 2013). These particular organs are most probably spared from adult monocyte infiltrates due to the combination of high self-renewal potential of theses specific tissue-resident macrophage populations with the restricted access to these locations for monocytes, since they are protected by the presence of the blood–brain and epithelial barriers. The appearance of monocyte-derived macrophages in tissue is accompanied by the gradual replacement of embryonic macrophages in either a quantitative fashion close to birth (Bain et al., 2014) or gradually progressing over time (Molawi et al., 2014; Sawai et al., 2016; Schulz et al., 2012). Monocyte-derived macrophages show significant gene modifications compared to their circulating equivalent as they adapt to the local tissue environment, and acquire a transcriptomic signature that shows similarities to resident macrophages of embryonic origin, even though epigenetic, transcriptional and functional differences remain (Cronk et al., 2018; Lavin et al., 2014; Scott et al., 2016b; T'Jonck et al., 2018; van de Laar et al., 2016). These newly recruited monocyte-derived macrophages have been shown to adopt functions of tissue-resident macrophages that they are replacing. This has been for example elegantly demonstrated for melanophages of the dermis that phagocytose skin pigments. The retention of pigment cargo in the skin is taken-over by newly recruited monocyte-derived melanophages, which helps explains tattoo persistence (Baranska et al., 2018). Whether monocyte-derived resident macrophages that engraft in steady-state tissues always acquire a self-maintenance capacity comparable to their embryonic counterparts is hotly debated and seems to depend on the tissue of residence. In contrast to this, monocyte-derived macrophages that engraft into the macrophage pool during inflammation show distinct gene signatures and often fail to self-maintain for prolonged periods of time (see below). The reason why monocyte-derived macrophages seed embryonic macrophage niches in particular tissues in steady state and how monocyte-derived cells efficiently compete with the embryonic populations remains largely unclear (Guilliams and Scott, 2017). It is possible that a low-grade tonic inflammation is required in order to recruit Ly6C^{Hi} monocytes to the tissues, since germ-free mice show significantly reduced numbers of monocytes and monocyte-derived macrophages in the intestine (Bain et al., 2014). Furthermore, continuous physical micro-trauma induced by muscle contraction like in the heart could hinder the survival of embryo-derived cardiac macrophages, thereby creating a niche for monocyte-derived cells (Molawi et al., 2014). A vacant cellular niche can also be induced experimentally by targeted genetic mutations or irradiation that influence the survival/proliferation of embryonic macrophages, while the continuously produced myeloid cells in the BM are spared of these effects and successfully compete with the affected embryonic derived population (Cronk et al., 2018; Varol et al., 2017).

On the other hand, current research also proposes that Ly6C^{Hi} monocytes can retain their monocyte-like state within tissues without further differentiation into macrophages. These emigrated Ly6C^{Hi} monocytes act as a local monocyte reservoir and show minimal transcriptional changes (Swirski et al., 2009) and might adopt antigen presentation functions (Jakubzick et al., 2013; Jakubzick et al., 2017), although their exact contribution to T cell priming *in vivo* and their time of residence in the tissue remains to be elucidated.

An alternative maturation route for Ly6C^{Hi} monocytes, rather than leaving the circulation to develop into specific tissue macrophages or a local monocyte reservoir, is the transition into Ly6C^{Low} monocytes within the circulation. This is associated with the establishment of *de novo* enhancers and the activation of poised enhancers that facilitate the transition into non-classical Ly6C^{Low} monocytes (Mildner et al., 2017; Polletti and Natoli, 2017). Mathematical modelling of the conversion rate of classical monocytes to non-classical monocytes based on pulse labelling decay in comparison to the extravasation rate into tissues indicates that the vast majority (>90%) of classical monocytes are recruited to peripheral tissues where they potentially differentiate into monocyte-derived macrophages or enter reservoirs of undifferentiated monocytes (Patel et al., 2017; Tak et al., 2017a). Interesting questions arise from these data. Do tissue infiltrated Ly6C^{Hi} monocytes under physiological conditions sometimes convert to Ly6C^{Low} monocytes and re-enter the circulation, or do these cells always differentiate into macrophages? If the latter is the case, how is the default transition program from Ly6C^{Hi} to Ly6C^{Low} monocytes prevented to allow alternative macrophage development and how do Ly6C^{Hi}-derived macrophages transcriptionally relate to Ly6C^{Low} monocytes? If monocytes remain as undifferentiated cells in specific tissue reservoirs, is there an active signal required to maintain them in this undifferentiated stage? Data to date indicates that infiltrating Ly6C^{Hi} monocytes show a high degree of differentiation potential, depending on the local tissue microenvironment, suggesting multiple cellular fates for tissue infiltrated Ly6C^{Hi} monocytes.

The plastically of the Ly6C^{Hi} monocyte population ignites a number of different scenarios. It is conceivable that Ly6C^{Hi} monocytes may represent a heterogeneous cell population with some cells primed for tissue infiltration and some cells primed for the transition into Ly6C^{Low} monocytes. Theoretically, the distinct circulating Ly6C^{Hi} monocyte subsets could even originate from different precursor cells. However, single-cell RNA sequencing of murine monocytes indicates that circulating Ly6C^{Hi} and Ly6C^{Low} monocytes represent relatively homogenous populations under physiological conditions (Mildner et al., 2017), even though a higher sequencing depth and the analysis of a greater number of monocytes might uncover additional monocyte subsets. Nevertheless, the intermediate subsets of Ly6C^{Int} and CD14^{+} CD16^{+} cells exhibit a certain degree of heterogeneity, in both mice and humans (Mildner et al., 2017; Villani et al., 2017). Specifically, murine Ly6C^{int} monocytes consists of two distinct populations, one of which is characterized by the C-type lectin receptor CD209a (DC-SIGN) and MHCII gene family expression, while the other subset can be interpreted as the “true” Ly6C intermediate monocyte subset, since they represent a transcriptional landscape linking classical Ly6C^{Hi} cells to non-classical Ly6C^{Low} monocytes (Mildner et al., 2017). The CD209a^{+} MHCII^{+}

Ly6C$^{Hi\text{-}to\text{-}int}$ monocytes are proposed to give rise to monocyte-derived DC under pathological conditions (Menezes et al., 2016). The origin of these cells is currently uncertain, even though these cells appear to develop independently of the transcription factor C/EBPβ (Mildner et al., 2017) or could represent cells that re-entered the circulation following secondary lymphoid tissue infiltration (Jakubzick et al., 2013). As mentioned, human CD14^{+} CD16^{+} monocytes likewise show a certain degree of heterogeneity (Villani et al., 2017) and are characterized in general by higher HLA gene family expression (Gren et al., 2015; Patel and Yona, 2018; Patel et al., 2017; Schmidl et al., 2014; Zawada et al., 2011). However, whether the murine CD209a-expressing Ly6C^{int} cells have a homologous human equivalent remains unclear, since the newly identified human CD14^{+} CD16^{+} subset is characterized by a cytotoxic gene signature, while this signature was absent in the two murine Ly6C^{int} populations (Villani et al., 2017).

Taken together, Ly6C^{Hi} monocytes represent a particularly versatile biological system that even during homeostasis provides various tissues, including the circulation system, with monocyte-derived cells that acquire incredibly diverse functions dependent on their tissue of residence.

Development of non-classical monocytes

Classical monocytes have a relatively short lifespan in the circulation of approximately one day; non-classical monocytes display a longer lifespan of around 2 days in mice and 7 days in humans, respectively (Patel et al., 2017; Yona et al., 2013). This can be indirectly observed in parabiotic mice, in which two mice are surgically joined and share a common circulation. In this system, donor-derived Ly6C^{Hi} monocytes do not reach equilibrium with their host Ly6C^{Hi} monocyte counterparts, in a similar manner to short-lived neutrophils, while lymphocytes and Ly6C^{Low} monocytes do (Liu et al., 2007). Interestingly, the lifespan of Ly6C^{Low} monocytes can be extended to up to 2 weeks, which is likely dependent upon the availability of CSF1 (Yona et al., 2013), a critical factor controlling monocyte/macrophage maintenance (Bartocci et al., 1987; Elmore et al., 2014). Possibly due to the increased lifespan of Ly6C^{Low} monocytes compared to their Ly6C^{Hi} counterparts, these cells are more susceptible to anti-CSF1R treatment or tamoxifen-induced deletion of the *Csf1r* gene. In both cases, the number of circulating Ly6C^{Low} monocytes decrease, while Ly6C^{Hi} monocyte numbers remain unaffected (Greter et al., 2012; Hoeffel et al., 2015; MacDonald et al., 2010; Mrdjen et al., 2018). The flexibility in increasing the lifespan of non-classical monocytes might secure their constant cell numbers even under pathological conditions, when the majority of classical monocytes are recruited to peripheral inflammatory lesions or when their functional transition into non-classical monocytes is blocked, e.g. due to inflammatory stimuli. This might also explain the finding that *Ccr2*-deficient mice show approximately the same cell numbers of non-classical monocytes compared to their wild type counterpart, since only a minor fraction of Ly6C^{Hi} monocytes is required to replenish a long-lived population (Yona et al., 2013). Alternatively, Ly6C^{Hi} monocytes trapped in the BM of *Ccr2*-deficient mice may convert locally into non-classical monocytes and leave the BM in a CCR2-independent

manner. Interestingly, in wild type/CCR2$^{-/-}$ mixed BM chimeras, where half the reconstituted BM is of wild type origin and the other half of CCR2$^{-/-}$, circulating Ly6C^{Low} monocytes are found to have the same chimerism as Ly6C^{Hi} monocytes and derive mainly from wild type cells (Yona et al., 2013), indicating that Ly6C^{Low} monocyte are also partially dependent on CCR2 signalling. Additional factors influencing Ly6C^{Low} monocyte survival include the chemokine CX_3CL1 (Landsman et al., 2009) and, surprisingly, TNF (Wolf et al., 2017).

The transition of classical monocytes to non-classical monocytes has been observed with the aid of the ablation of circulating monocytes by clodronate-loaded liposomes, cell transfer and pulse labelling experiments in different species including the mouse (Sunderkotter et al., 2004; Tacke et al., 2006; Varol et al., 2007; Yona et al., 2013), rat (Yrlid et al., 2006), macaque (Sugimoto et al., 2015) as well as in humans (Patel et al., 2017) and therefore seems to present a evolutionary conserved developmental program. However, the identification of cellular conversion from classical to non-classical monocytes does not exclude that some cells in the non-classical monocyte pool may develop without passing through a classical monocyte stage (Carlin et al., 2013).

The mechanisms driving the conversion of Ly6C^{Hi} monocytes into Ly6C^{Low} monocytes are now better understood. Delta-like 1 (Dll1) signals from endothelial cells that interact with NOTCH2 on Ly6C^{Hi} monocytes facilitates the conversion into Ly6C^{Low} cells (Gamrekelashvili et al., 2016). These data indicate that Ly6C^{Hi} and Ly6C^{Low} monocytes are biologically interconnected, a fact that is also observed on the epigenetic level, since both monocyte subsets take advantage of the same promoter repertoire and only show little differences in chromatin organisation (Mildner et al., 2017). The differences, which can be detected and arise during the Ly6C^{Hi} to Ly6C^{Low} transition, are due to the establishment of *de novo* enhancers and activation of enhancers by histone acetylation (Mildner et al., 2017; Thomas et al., 2016).

On the molecular level, the conversion from classical to non-classical monocytes is accompanied by up-regulation of the transcription factors C/EBPβ, NR4A1 and KLF2. Absence of NR4A1 leads to reduced Ly6C^{Low} monocyte survival and increased apoptosis (Hanna et al., 2011). Further epigenetic experiments identified one particular *Nr4a1* enhancer (E2), which is specific to monocytes and the sole deletion of this small enhancer element leads to the absence of Ly6C^{Low} monocytes (Thomas et al., 2016). The transcription factors KLF2 and C/EBPβ can bind to this E2 enhancer and induce Nr4a1 expression. Subsequently, both genes were shown to be involved in Ly6C^{Low} monocyte generation (Hanna et al., 2011; Mildner et al., 2017; Tamura et al., 2017; Thomas et al., 2016). Also *Csf1r* is regulated by C/EBPβ and therefore it is likely that the absence of Ly6C^{Low} monocytes in C/EBPβ deficient mice is in part due to their inefficiency to up-regulate *Csf1r* and *Nr4a1* expression (Mildner et al., 2017; Tamura et al., 2017). Although it remains to be experimentally demonstrated that human monocytes follow a similar molecular program during transition, the transcriptional network analysis strongly supports C/EBPβ as a central player in human monocyte biology (Schmidl et al., 2014). Moreover, C/EBPβ as well as NR4A1 motif enrichment are evident in human bone marrow

monocytes by single-cell ATAC sequencing (Buenrostro et al., 2018; Buenrostro et al., 2017). Note that while single-cell RNA sequencing revealed that Ly6C^{Low} monocytes are relatively homogenous population (Mildner et al., 2017), it is plausible that a distinct subset(s) of Ly6C^{Low} patrolling monocytes strongly adhere to the endothelium and are not retrieved by conventional bleeding regimes.

The ratio between classical and non-classical monocytes is dynamic and influenced by various signals with distinct outcomes on monocyte numbers and subset distribution. The proportion of circulating classical and non-classical monocytes varies dependent on monopoiesis, tissue infiltration or the emergency release of these cells from central (BM) or peripheral reservoirs. For instance, mice treated with the bacterial peptidoglycan muramyl dipeptide show increased conversion rate of Ly6C^{Hi} monocytes into Ly6C^{Low} cells in a NOD2-dependent mechanism (Lessard et al., 2017), while low concentrations of Toll-like receptor (TLR) ligands in the bloodstream of mice drive CCR2-dependent emigration of monocytes from BM to the circulation and thereby lead to increased circulating classical Ly6C^{Hi} monocyte cell numbers (Shi and Pamer, 2011). In humans endotoxin induces a rapid yet transient monocytopenia during the first two hours after lipopolysaccharide (LPS) injection followed by the sequential reappearance of CD14^{+} CD16^{-} classical monocytes, followed by CD14^{+} CD16^{+} intermediate cells and finally CD14^{Low} CD16^{+} non-classical monocytes (Patel et al., 2017; Tak et al., 2017b; Thaler et al., 2016). Also exercise and age can also influence the dynamic equilibrium of monocyte subsets (Heimbeck et al., 2010; Seidler et al., 2010; Verschoor et al., 2014). The number of non-classical monocytes is thus strongly linked to the physiological status of the organism and therefore represents a potential diagnostic tool during certain pathological conditions (Selimoglu-Buet et al., 2015).

Function of non-classical monocytes in healthy homeostasis

The function of non-classical monocytes is emerging: intravital microscopy studies have revealed that Ly6C^{Low} monocytes continuously monitor the vasculature under physiological conditions through an LFA/ICAM-dependent crawling mechanism on the resting endothelial cells (Auffray et al., 2007; Carlin et al., 2013). This patrolling behaviour of Ly6C^{Low} monocytes can be observed in capillaries, arterioles and venules. Similarly, human CD14^{Low} CD16^{+} non-classical monocytes show patrolling behaviour when adoptively transferred into immuno-compromised mice (Cros et al., 2010). This crawling characteristic permits Ly6C^{Low} monocytes to efficiently scavenge luminal microparticles under physiological conditions and they also play a key role in the surveillance of endothelial cell integrity.

Extramedullary haematopoiesis and monocyte reservoirs

The BM has long been established as the *bona fide* organ for adult monocyte generation but, interestingly, a fraction of murine monocyte-committed precursors, e.g. MDP and cMoP, were detected in the spleen under steady healthy homeostasis (Hettinger et al., 2013). Since no circulating

monocytic precursor could be detected in the blood (Hettinger et al., 2013), BM-independent splenic production of monocytes is a plausible option. The spleen serves as a significant reservoir for Ly6C^{Hi} as well Ly6C^{Low} monocytes; remarkably these monocyte pools outnumber their circulating counterparts (Swirski et al., 2009). In addition, the lung and the skin have been proposed to harbour a pool of undifferentiated monocytes and might serve as reservoirs as well (Jakubzick et al., 2013). Under certain inflammatory circumstances, these monocytes are released into the circulation and the emergency extramedullary monopoiesis is augmented in an IL-1β-dependent mechanism (Leuschner et al., 2012). Mobilized classical monocytes from the spleen are rapidly recruited to injured tissue as observed in models of atherosclerosis (Robbins et al., 2012) and ischemic myocardial injury (Swirski et al., 2009; van der Laan et al., 2014). The precise contribution of monocytes released from peripheral reservoirs to the pool of monocytes recruited to inflamed tissues remain largely unknown and additional research is required to uncover the precise role of these monocyte reservoirs.

Pathological conditions and emergency monopoiesis

Inflammation requires the rapid recruitment of short-lived myeloid cells to sites of injury, a process that relies on the constant generation and mobilization of BM cells. Therefore, severe inflammation can induce a state of emergency that may generate monocytes with alternative antecedents to steady-state. These include monocytes that have bypassed the canonical MDP-cMoP-monocyte developmental pathway and resemble neutrophil-like Ly6C^{Hi} monocytes that derive from the GMP ((Yanez et al., 2017); Fig. 3). Another example of a recently described monocyte subset that appears under inflammatory conditions are the segregated nucleus-containing atypical Ly6C^{Low} monocytes ((SatM) (Satoh et al., 2017). SatM and neutrophil-like Ly6C^{Hi} monocytes appear to represent a negligible fraction of monocytes in steady-state (Mildner et al., 2017; Satoh et al., 2017; Yanez et al., 2017), yet become conspicuous during inflammation (Satoh et al., 2017; Yanez et al., 2017). In the case of neutrophil-like Ly6C^{Hi} monocytes it has been proposed that LPS challenge favours GMP-derived neutrophil-like Ly6C^{Hi} monocytes production while CpG challenge favours classical MDP-derived monocyte production (Yanez et al., 2017). At present we lack reliable surface markers to distinguish neutrophil-like Ly6C^{Hi} monocytes (identified using GFI1/IRF8-reporter mice, (Yanez et al., 2017)) from classical Ly6C^{Hi} monocytes and SatM (identified as Ly6C^{Low} Ceacam1^{Hi} Msr1^{Hi}, (Satoh et al., 2017)) from patrolling Ly6C^{Low} monocytes. Furthermore, mice infected with *Toxoplasma gondii* are characterized by a shift in their monocytic phenotype. These IFN-γ induced MHCII^{Hi} Sca-1^{Hi} monocytes were shown to produce more IL-10 as well as prostaglandin E2 (PGE_2) and were proposed to be of a regulatory phenotype (Askenase et al., 2015).

In conclusion, emergency monopoiesis does not only induce a shift in number of monocytes produced but also affects their function and phenotype. Because of the functional specialization of these newly identified monocyte subsets such as the CD209a^{Hi} MHCII^{Hi} monocytes, the SatM and the neutrophil-like Ly6C^{Hi} monocytes, it will be interesting to study emergency monopoiesis at the single-cell level

to assess whether it is associated to increased output of Ly6C^{Hi} monocytes with an altered function or whether it is based on the development of monocyte subsets through altered developmental pathways (e.g. neutrophil-like Ly6C^{Hi} monocytes or SatM), yielding cells that are not normally present in the steady-state and are functionally distinct from classical Ly6C^{Hi} monocytes.

From emergency haematopoiesis to trained immunity

In the recent years it has become clear that cytokines not only induce functional and phenotypical changes in monocytes directly but also influence the cellular outcome of haematopoietic stem cells. Therefore emergency haematopoiesis during infections can have long-lasting effects characterized by a shift in cell fate resulting in the higher production of particular immune cell types at the expense of other cells, such as the increased production of monocytes compared to the decreased production of DC (Pasquevich et al., 2015) and lymphocytes (Liu et al., 2015; Maeda et al., 2009; Pietras et al., 2016) (Fig. 3). This can result in increased numbers and an altered activation state of monocytes even weeks after the pathogen clearance. This phenomenon has been termed "trained immunity" (Netea et al., 2016; Quintin et al., 2012). A number of cytokines have been proposed to play a key role in trained immunity, including IFN-γ and IL-1β, suggesting that activation of stem cells by inflammatory cytokines produced by immune or non-immune cells in the BM is crucial for these long-lasting training effects (for review see: (Boettcher and Manz, 2017)). There is however no doubt that the stem cells are characterized by a "trained" cellular status through epigenetic changes since haematopoietic stem cells isolated from animals injected with β-glucan preserved their capacity to generate more myeloid cells 12 weeks post transfer into untrained animals (Mitroulis et al., 2018). Similar findings have been reported in mice trained by Bacillus Calmette-Guérin (BCG), accompanied by the increased presence of myeloid biased MMP in the BM (Kaufmann et al., 2018). Trained phenotypes not only comprise quantitative effects on myeloid cells, but also functional aspects. Macrophages differentiated from BCG-trained BM cells possessed an increased capacity to kill *Mycobacterium tuberculosis in vitro* and upon intratracheal transfer *in vivo*. Moreover, parabiotic partners of trained animals were better protected against *Mycobacterium tuberculosis* demonstrating that circulating cells (presumably monocytes) and not tissue resident cells from the trained partner can mediate increased protection (Kaufmann et al., 2018). Trained immunity also occurs during inflammation associated with metabolic diseases. *Ldlr*$^{-/-}$ mice put on western high-fat diet, a standard model for atherosclerosis, develop systemic inflammation. When these mice were put on western diet for 4 weeks and then returned to control diet for additional 4 weeks, serum cytokines dropped back to steady-state levels, while BM and splenic myeloid cells produced more inflammatory cytokines upon TLR stimulation compared to control mice (Christ et al., 2018).

The concept of trained immunity has been translated to humans. BCG vaccination induced epigenetic changes in circulating monocytes of healthy volunteers 4 weeks after vaccination (Arts et al., 2018). BCG-vaccinated individuals displayed lower circulating viral titters upon subsequent experimental

viral infection and their PBMCs produced higher levels of IL-1β, TNF and IL-6 upon stimulation *in vitro*. Importantly, no transcriptomic changes were observed in monocytes in these subjects, highlighting the need to study epigenetic modifications when examining trained immunity.

Taken together, these studies demonstrate that inflammation-induced emergency haematopoiesis – with IL-1β and IFNs emerging as the main drivers – can result in trained immunity characterized by long term epigenetic effects on stem cells to generate higher quantities of monocytes possessing increased reactivity to various TLR stimulations and superior killing activities against pathogens. The epigenetic changes associated with monocyte training involve histone modifications of genes encoding pro-inflammatory cytokines such as IL-6 and TNF but also of genes involved in the mTOR pathway (Netea et al., 2016; Quintin et al., 2012). Changes in the mTOR metabolic pathway seem essential for monocyte training since inhibition of mTOR reduced the increased TNF production associated with training (Cheng et al., 2014).

The particular factors involved in the epigenetic modification of stem cells and whether training of stem cells is also associated with an increased production of particular subsets of monocytes is currently unknown (Fig. 3). It will also be important to experimentally demonstrate the intrinsic effect of training on the function of monocyte-derived cells. Indeed, in many training experiments it is difficult to distinguish between the indirect effects of prior inflammatory reactions (effect on local stromal cells, resident macrophages or memory lymphocytes) from the direct effect on BM stem cells and their trained monocyte progeny.

Function of classical monocytes during pathology

Under pathological conditions monocytes gain distinct non-redundant functions that often cannot be fulfilled by resident macrophages and DC. These non-exclusive and sometimes overlapping effector functions comprise pro-inflammatory activities, antigen presentation (monocyte-derived DCs) and tissue remodelling / anti-inflammatory abilities (Fig. 4).

The contribution of monocytes during inflammation

The function and importance of pro-inflammatory monocytes is well documented in autoimmune diseases, such as the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), rheumatoid arthritis, colitis and bacterial infections (Udalova et al., 2016). During these diseases, $Ly6C^{Hi}$ monocytes are mobilised from the circulation or peripheral reservoirs, resulting in the rapid influx of classical monocytes into the affected tissue or organ. Initial experiments with CCR2-deficient mice that lack most circulating classical monocytes showed that these mice are virtually resistant to EAE induction (Fife et al., 2000; Izikson et al., 2002), a phenotype that could be later attributed to reduced numbers of pro-inflammatory monocytes in this mouse strain and to the pathogenic function of monocytes during disease development (Ajami et al., 2011; King et al., 2009; Mildner et al., 2009). It was also shown that while infiltrated monocytes show an inflammatory

phenotype in EAE, tissue resident microglia on the other hand were phagocytotic active and did not show an overt pro-inflammatory gene signature (Yamasaki et al., 2014), indicating a division of labour between these myeloid subsets.

Even though EAE is considered to be a T cell-mediated disease, all attempts to identify classical effector T cell-secreted cytokines with encephalogenic activities like IFN-γ, IL-17, IL-22 and IL-12 failed (reviewed in (Becher and Segal, 2011)). However, mice lacking *Csf2*, which is secreted in a RORγt-dependent manner by $CD4^+$ T cells (Codarri et al., 2011), are completely protected to EAE induction (McQualter et al., 2001). Even though CSF2 is not directly involved in monocyte generation (Hibbs et al., 2007), CSF2 can determine the functional outcome of monocyte-derived cells under pathological conditions. Using CCR2-CreERT2 mice it was demonstrated that CSF2R-signaling is needed for the production of inflammatory cytokines and chemokines by monocyte-derived cells in EAE (Croxford et al., 2015). Furthermore, the sole overexpression of CSF2 by T cells renders monocytes towards an $MHCII^+$ $CD11c^+$ phenotype and leads to CNS infiltration and CNS tissue damage in an antigen-independent manner (Spath et al., 2017). A comparable CNS pathology could also be observed in mice receiving peripheral FLT3L injection in combination with the transgenic overexpression of CCL2 in oligodendrocytes, which was also T cell independent (Furtado et al., 2006). Since FLT3L injection also increases the number of circulating monocytes (Furtado et al., 2006; Mrdjen et al., 2018), the observed phenotype in these mice might be in part similarly mediated by pathogenic monocytes.

Similar to the situation in EAE, *Ccr2*-deficient mice are protected in dextran sulphate sodium (DSS)-induced colitis, a model of ulcerative colitis (Platt et al., 2010), which indicates a similar detrimental role for monocyte-derived effector cells (Zigmond et al., 2012). However, colitis does not rely on CSF2 signalling since *Csf2*-deficient mice are more susceptible to DSS-induced colitis (Xu et al., 2008), while CSF2 administration ameliorates clinical symptoms and accelerates colonic tissue repair (Bernasconi et al., 2010). These data are in accordance with the observation that T cell-restricted overexpression of CSF2 does not lead to any monocytic infiltration in the intestine (Spath et al., 2017). Effector monocytes recruited during DSS-colitis, produced less IL-10 and more TNF than resident monocyte-derived macrophages that developed before the onset of colitis (Bain et al., 2013). This lack of IL-10 production may further drive colitis although it is the IL-10 produced by regulatory T cells, but not resident monocyte-derived macrophages that is crucial to maintain homeostasis in the colon. One of the main functions of intestinal IL-10 is to suppress monocyte-derived cells themselves as lack of IL-10R on intestinal myeloid cells leads to spontaneous colitis (Zigmond et al., 2014a).

Another subset of effector monocytes are the TNF/iNOS-producing (Tip)-DC, which were originally identified in the spleen of mice during *Listeria monocytogenes* infection (Serbina et al., 2003), but could also detected in mice infected with the parasite *Trypanosoma brucei* (Bosschaerts et al., 2010; Guilliams et al., 2009), *Toxoplasma gondii* (Dunay et al., 2008) or *Leishmania major* (De Trez et al., 2009). TipDCs derive from $Ly6C^{Hi}$ monocytes (Guilliams et al., 2009; Serbina et al., 2003) and are

characterized by the surface marker expression $CD11c^{Int}$ $MHCII^{+}$ $Ly6C^{Int}$ and by their high production of TNF and nitric oxide. Although these cells were classified as "DC" due to their considerable CD11c and MHCII expression, their contribution to $CD4^{+}$ T cell priming during *Listeria* infection appears negligible (Serbina et al., 2003). The term TipDC is therefore misleading and should be avoided in our view. Rather these cells excel in the killing of pathogens as demonstrated by the much higher *Listeria*, *Toxoplasma* and *Leishmania* burden in mice lacking recruited monocytes (De Trez et al., 2009; Dunay et al., 2008; Serbina et al., 2003), highlighting that the resident macrophage pool is not sufficient to clear these pathogens and require assistance from recruited effector monocytes. Note that the accumulation of effector monocytes during infections can also significantly contribute to collateral tissue damage (Guilliams et al., 2009).

It was recently reported that effector monocytes actively participate in the development of fibrosis. This involves the specific subset of GMP-derived C/EBPβ-dependent SatM that produce high levels of TNF. Mice lacking C/EBPβ were protected from bleomycin-induced fibrosis and the transfer of wild type SatM was sufficient to restore fibrosis development (Satoh et al., 2017). Monocytes also differentiate into pathogenic foam cells in the atherosclerotic plaques (Hilgendorf et al., 2015). Anitschkow (Dock, 1958) demonstrated that by feeding rabbits purified cholesterol caused vascular changes leading to the formation of lesions and the presence of cholesterol-laden cells (foam cells) similar to those detected during atherosclerosis in humans. Now we know that foam cell in the atheroma are descendants of selectively recruitment classical monocytes. These monocytes accumulate in the growing lesions, take-up excess lipid particles and further exacerbate the disease by producing inflammatory cytokines and chemokines that attract additional monocytes. CCR5 and CX_3CR1 appear to be involved in their retention within the atherosclerotic plaque (Tacke et al., 2007) and it was demonstrated that foam cells also proliferate extensively within the plaque (Robbins et al., 2013).

These examples highlight how recruited monocytes play an essential role as an emergency squad, acquiring strong microbicidal and pro-inflammatory activity. Monocytes are therefore not simply precursors that get recruited to temporarily increase the number of resident macrophages. Monocyte-derived macrophage recruited in inflamed tissues are in fact functionally distinct from the resident macrophages and are crucial for host defence, but at the same time can significantly exacerbate clinical symptoms by inducing collateral tissue damage. Nevertheless, in certain situations, such as helminth infections, IL-4 can drive the proliferation and the immune response of resident macrophages without the aid of recruited monocytes (Jenkins et al., 2011).

Monocytes and antigen presentation

Monocytes can acquire antigen-presentation capacities and display a DC-like phenotype. This was initially observed when human blood myeloid cells were stimulated with CSF2 alone or in combination with IL-4, which promotes the differentiation into $CD11c^{+}$ $MHCII^{+}$ $CD11b^{+}$ cells with

soluble antigen presenting capacity *in vitro* (Sallusto and Lanzavecchia, 1994). These monocyte-derived DC could be obtained by culturing murine BM cells with CSF2 (Inaba et al., 1992). However, it was recently shown that CSF2-treated BM cultures consist of a heterogeneous cell population developing from distinct precursors, including conventional DC precursors (Helft et al., 2015). Therefore, in order to evaluate the antigen-presentation capacity of monocyte *in vitro*, it is advisable to FACS sort pure monocytes as a starting population. Mouse monocytes cultured in presence of IL-4 and CSF2 were shown to cross-present cell-associated antigen efficiently (Briseno et al., 2016). These monocyte-derived DC expressed high levels of BATF3 and IRF4. Interestingly, *Batf*-triple deficient cells, but not *Irf4*-deficient cells, still cross-presented antigen thereby demonstrating that cross-presentation is controlled by distinct transcriptional programs in monocyte-derived DC as compared to *Batf*-dependent cDC1, which do not require IRF4 for this function. IRF4 was also reported to be essential for the IL-4 driven development of monocyte-derived DC from human monocytes *in vitro* (Goudot et al., 2017). Comparable to the situation in mice, high-dimensional mass cytometry analysis revealed that *in vitro* cultures of human monocytes with CSF2 and IL-4 yield cells with important phenotypic heterogeneity (Sander et al., 2017). This could potentially be the result of different human monocyte subsets that are present in the circulation (see above). Indeed in mouse it was found that a small subset of $CD209a^+$ monocytes possess a greater potential to differentiate into monocyte-derived DC (Menezes et al., 2016). It is possible that this population is boosted by CSF2 treatment.

It has been challenging to assess the contribution of monocyte-derived DC to antigen-presentation *in vivo.* For an overview of the studies proposing a role of monocyte-derived DC in antigen-presentation we redirect the reader to a recent review on the subject (Jakubzick et al., 2017). Multiple studies have demonstrated that monocytes can take up, process and present antigen *in vivo* but whether this contribution is significant to T cell priming compared to antigen-presentation by classical DC is debatable and seems to depend on the inflammatory context. Unfortunately, it has not been possible to restrict antigen-presentation to monocyte-derived DC or to specifically knock-down antigen-presentation in these cells *in vivo*. Moreover, we still lack reliable *in vivo* markers to distinguish activated cDC2 from monocyte-derived DC and, finally, it is not clear whether *in vivo* monocyte-derived DC differentiate solely from $CD209a^+$ monocytes or whether multiple monocyte subsets have the ability to acquire a monocyte-derived DC profile in tissues.

Contribution of monocytes during tissue remodelling

Inflammation is fundamental to the survival of an organism, as it ensures defence against harmful external stimuli but also repair to injured tissue. The resolution phase of the inflammatory response is not a passive process – the objective of this phase is to re-establish the tissue back to healthy homeostasis (Hunter, 1794) and if this resolution phase is disrupted, chronic inflammatory diseases can occur. Mononuclear phagocytes help orchestrate a number of aspects of the resolution phase: for example, the safe disposal of cell debris, cytokine catabolism and the release of regulatory cytokines

and eicosanoids (Newson et al., 2014). Monocytes can already acquire a regulatory phenotype before reaching the injured or infected tissue whist still in the circulation (Askenase et al., 2015).

In a model of resolving myocardial infraction, CCR2-expressing infiltrating monocytes are thought to promote tissue remodelling and repair via expression of VEGF that induces angiogenesis and by promoting myofibroblast accumulation (Nahrendorf et al., 2007), and this reparative behaviour is dependent on NR4A1 (Hilgendorf et al., 2014). In paracetamol-induced liver injury infiltrating monocytes are essential for hepatic regeneration and clearance of neutrophils (Graubardt et al., 2017; Zigmond et al., 2014b). This anti-inflammatory phenotype was also adopted during CCl_4-induced liver fibrosis, where infiltrating Ly6C^{Hi} monocytes recruited to the fibrotic liver are responsible for the resolution of tissue fibrosis by degrading extracellular matrix, clearing cell debris and accelerating scar resolution (Ramachandran et al., 2012). Ly6C^{Hi} monocytes are also recruited to chronic allergic inflammed skin, where they differentiate in the presence of basophils and in an IL-4-dependent mechanism into an anti-inflammatory cell to dampen the allergic response (Egawa et al., 2013). In this context is was also shown that IL-4-activated monocyte-derived F4/80^{int} CD206^{+} macrophages undergo in the presence of vitamin A a conversion into macrophages with a tissue-resident F4/80^{hi} CD206^{-} phenotype in the peritoneal cavity of mice (Gundra et al., 2017). A similar mechanism was also observed during the formation of liver granulomas in mice infected with *Schistosoma mansoni* (Gundra et al., 2017). Monocytes recruited to the retina injury play an essential role in the survival and proliferation of retinal progenitor cells by the production of high levels of IL-10 (London et al., 2011). In a model of spinal cord recovery following the centralised injury of the spinal cord, recruited Ly6C^{Hi} monocyte adopt an anti-inflammatory phenotype and secret IL-10, possibly counteracting the detrimental activity of resident microglia (Shechter et al., 2009). This highlights the regulatory role monocyte possess, which can dampen the activation of resident macrophages. Finally, development of monocyte-derived macrophages with a tissue remodelling phenotype following injury is not always binary (with monocytes differentiating in either pro- or anti-inflammatory macrophages). For instance, injection of the tiger snake venom notexin, a myotoxin phospholipase leads to the destruction of skeletal muscle, is accompanied by the infiltration of Ly6C^{Hi} monocytes that first display an early pro-inflammatory phenotype, before converting into a reparative monocyte-derived macrophage with the capacity to stimulate myogensis, fibre growth and restore muscle integrity (Arnold et al., 2007). Taken together, these studies highlight the plasticity of monocytes and demonstrate that these cells are essential for tissue repair and perform tasks that are not efficiently performed by resident macrophages.

Role of monocytes in cancer

Mononuclear phagocytes are located in both healthy and pathological tissue. The tumour microenvironment is no exception and the contribution of monocytes to tumour development is a multifaceted process ranging from the initiation of vessel growth to immune escape and metastasis. Tumours circumvent recognition and cell mediated elimination by suppressing the anti-tumor immune response. In this regard, it was shown that tumours affect myelopoiesis in the BM and induce the

expansion of myeloid cells with immunosuppressive activity, in both animal models and human patients (Bronte et al., 2016). These cells were originally characterized by CD11b and Gr1 expression and termed myeloid-derived suppressor cells' (MDSC). However, CD11b and Gr1 (which recognizes both Ly6C and Ly6G) define classical monocytes as well as neutrophils and therefore both myeloid cell populations could potentially contribute to T cell suppression. Analysis of this $CD11b^{+}$ $Gr1^{+}$ subset as a homogenous population has caused conflicting results and confusion concerning the nature, function and ontogeny of these cells. The addition of further cell surface markers unmasked the presence of monocytic and polymorphonuclear MDSC and allowed the independent analysis of both subsets (Bronte et al., 2016; Gallina et al., 2006; Huang et al., 2006; Movahedi et al., 2008). Subsequent adoptive transfer experiments and molecular analysis identified that monocytic MDSC are derive from circulating $Ly6C^{Hi}$ monocytes, either directly from the BM or from the splenic peripheral reservoir in a CCR2-dependent manner (Cortez-Retamozo et al., 2012; Shand et al., 2014). The presence of hypoxic conditions, high concentrations of oxidative agents, the release of pro-inflammatory cytokines, and limited supply of nutrients makes the tumour environment a relatively harsh milieu. Monocytes, which infiltrate the tumour tissue under these circumstances, might consequently acquire a pro-inflammatory signature that dampens lymphocyte activities, survival and proliferation in general – a phenomenon reflected in the suppressive activities of MDSC. However, these infiltrating $Ly6C^{Hi}$ monocytes can further differentiate into tumour-associated macrophages (TAM), which are – similar to the situation in other pathological contexts – characterized by low Ly6C expression and differential expression of MHCII, CD11c and CX_3CR1 (Movahedi et al., 2010). In general, TAM represent the majority of tissue-resident macrophages in tumours beside a small fraction of embryonic-derived macrophages (Franklin et al., 2014) but a differential role for embryonic macrophages have also been proposed
(Bowman et al., 2016; Zhu et al., 2017). TAM provide maintenance activities for tumour growth by facilitating angiogenesis, acting as pathfinders for endothelial cells, which provide oxygenation and nutrients to the growing tumour, as well as by clearing waste products. TAM exhibit negative activities on T cells and express high levels of programmed death ligand 1 (PD-L1), a ligand for immune-checkpoint receptor that restricts CD8 T cell activities (Noy and Pollard, 2014). Monocyte-derived TAM therefore represents an essential component of the developing tumours by critically contributing to survival, protection and growth of tumour cells. The development of monocytic MDSC and TAM is dependent on the transcription factors IRF8 (Waight et al., 2013), STAT3 (Chalmin et al., 2010), RBPJ (Franklin et al., 2014) and CEBP/b (Marigo et al., 2010).
Finally, the majority of deaths from solid tumours are a result of metastasis. Monocytes promote metastasis by preparing the potential microenvironment and enable the dissemination of metastatic cells (Keirsse et al., 2018). Classical monocytes are recruited to the metastatic site in a CCL2-CCR2 dependent fashion and, once embedded within this niche, these recruited monocyte-derived cells promote the survival and growth of the metastatic tumour (Qian et al., 2011).

In conclusion, whilst it is clear that monocyte-derived cells play an important role in cancer, many confusing and out-dated concepts (like the MDSC and M1 versus M2 tumor associated macrophages) have been plaguing the cancer literature. In the end, tumour mononuclear phagocyte biology should be analysed like any other pathological tissue with the potential of diverse roles for resident macrophages versus recruited monocytes, with the possible existence of particular pre-committed monocyte subsets with specific functional specialization, and with the potential existence of distinct micro-environments or "niches". The advance of fate mapping tools, single-cell sequencing, spatial transcriptomics and multiplexed microscopy will surely shed light on the precise role for monocytes in cancer and will hopefully yield novel anti-cancer strategies.

Function of non-classical monocytes during pathology

The function of Ly6C^{Low} monocytes during inflammation remains controversial. The existence of Ly6C^{Hi} and Ly6C^{Low} monocytes within the injury site was originally interpreted as evidence that both classical and non-classical monocytes can infiltrate the tissue in a sequential manner and might contribute distinct pro- and anti-inflammatory activities. Alternatively, infiltrated Ly6C^{Hi} monocytes can lose their Ly6C expression while increasing F4/80, CD11c and CX_3CR1 expression during the differentiation process into effector monocyte-derived cells in the tissue, making them indistinguishable from circulating Ly6C^{Low} monocytes (Arnold et al., 2007; Avraham-Davidi et al., 2013; Zigmond et al., 2012). Examining the phenotype of infiltrating cells at the site of injury and extrapolating their origin based on surface markers has therefore caused much confusion.

Further insight into the nature of non-classical monocytes came from the observation that *Nr4a1*-deficient mice are characterized by a strong reduction of circulating Ly6C^{Low} monocytes (Hanna et al., 2011). Therefore, this mouse model in combination with the CX_3CR1^{gfp} reporter mouse (Jung et al., 2000) has become a useful tool to study the functional role of Ly6C^{Low} monocytes during different pathological conditions. It was shown that these patrolling Ly6C^{Low} monocytes interact with metastatic tumour cells, scavenge tumour material from the lung vasculature, and initiate natural killer cell recruitment and activation (Hanna et al., 2015; Plebanek et al., 2017). Ly6C^{Low} monocytes are also able to target and eliminate amyloid-β within the lumen of veins and the absence of these cells due to *Nr4a1*-deficiency increased amyloid-β deposits within the cortex and hippocampus of mice (Michaud et al., 2013).

As a word of caution, Ly6C^{Hi} monocyte function is not completely independent of the transcription factor NR4A1, since Ly6C^{Hi} monocytes that infiltrate the infarcted myocardium show increased expression of NR4A1(Hilgendorf et al., 2014). Furthermore, up-regulation of this transcription factor was accompanied by reduced pro-inflammatory activities and a reparative phenotype, while absence of NR4A1 led to a more inflammatory phenotype in Ly6C^{Hi} monocyte derived cells, which results in defective healing and compromised heart function (Hilgendorf et al., 2014). Similar results were obtained in an animal model of atherosclerosis, in which *Nr4a1*-deficient monocytes and macrophages

displayed enhanced toll-like receptor signalling and a polarization towards a pro-inflammatory phenotype (Hanna et al., 2012). Therefore, it is difficult to interpret the phenotype observed in *Nr4a1*-deficient mice as being exclusively due the absence of Ly6C^{Low} monocytes. A new and elegant mouse model will help to resolve these uncertainties. Deletion of a monocyte-specific *Nr4a1*-enhancer element (E2) resulted in the loss of peripheral non-classical monocytes, while NR4A1-dependent inflammatory pathways were preserved in macrophages (Thomas et al., 2016).

Beside these complications in deciphering results from mice with an absence of Ly6C^{Low} monocytes, some specific functions during inflammation have been attributed to these cells. Following exposure to TLR7-mediated 'danger' signals, mimicking viral infection or local cell death, a CX_3CR1-dependent retention of Ly6C^{Low} monocytes on the affected endothelium was observed (Carlin et al., 2013). The TLR7 ligand triggered monocytes lead to the recruitment of neutrophils, which mediated focal endothelial necrosis, while subsequently Ly6C^{Low} monocytes remove cellular debris (Carlin et al., 2013). Recently it was shown that non-classical monocytes are superior producers of TNF following poly(I:C) injection (Garre et al., 2017). The secretion of TNF by these cells led to reduced learning-induced dendritic spine remodelling in pyramidal neurons and consequently to deficits in learning behaviour (Garre et al., 2017). The production of TNF in response to viruses was also shown for human non-classical monocytes (Cros et al., 2010) and it is possible that future research will indicate a specialisation of Ly6C^{Low} monocytes in anti-viral immunity. Finally, recently it was proposed that Ly6C^{Low} monocytes exhibit a protective effect during the early stages of atherogenesis by maintaining endothelial cell integrity (Quintar et al., 2017).

Engraftment of monocytes in the resident macrophage pool during pathology

Another way by which inflammation-induced monocytes possessing an altered activation state will exert long-lasting effects on tissues is by integrating into the resident macrophage pool (Bonnardel and Guilliams, 2018). In some disease models, but not in others, the macrophage disappearance reaction that is often associated with inflammation (Barth et al., 1995) results in the engraftment of monocytes in the resident macrophage niche (Guilliams and Scott, 2017). In the case of the liver as an example, experimental diphtheria toxin induced depletion (Scott et al., 2016b), *Listeria monocytogenes* infection (Bleriot et al., 2015), non-alcoholic steatohepatitis (Devisscher et al., 2017), overload of senescent red-blood cells (Theurl et al., 2016), chemical induced liver fibrosis (Ramachandran et al., 2012) and paracetamol induced liver-injury (Zigmond et al., 2014b) result in partial Kupffer cell loss. Subsequently, infiltrating monocytes can differentiate into Kupffer cells during all these experimental models apart from paracetamol-induced liver injury, which solely relies on the proliferation of the remaining Kupffer cells to refill this niche (Zigmond et al., 2014b). A recent report demonstrates that monocyte to Kupffer cell differentiation is suppressed by Type I IFN signalling during a model for viral hepatitis (Borst et al., 2017). The underlying mechanism is unclear. Is Type I IFN inducing the generation of BM inflammatory monocytes that are blocked from becoming Kupffer cells before

reaching the liver? Does Type I IFN change the local microenvironment in the liver and block monocyte to Kupffer cell development?

When monocytes enter the Kupffer cell pool during inflammation it is uncertain whether these cells are functionally distinct from embryonic Kupffer cells. Indeed, it may be that these cells have been trained by the inflammation present during their development and are therefore more reactive to subsequent stimuli. In some models these monocyte-derived Kupffer cells eventually disappear from the Kupffer cell pool (Devisscher et al., 2017; Theurl et al., 2016), suggesting a diminished self-maintenance capacity compared to embryonic Kupffer cells.

Gammaherpes virus infection has been associated with the generation of IL-10 producing MHCII^{Hi} Sca-1^{Hi} monocytes within the BM (Machiels et al., 2017). These monocytes resemble monocytes with a regulatory phenotype produced during *Toxoplasma gondii* infection (Askenase et al., 2015). Interestingly, gammaherpes virus infection also resulted in the engraftment of these regulatory monocytes in the alveolar macrophage pool. These virus-induced monocyte-derived alveolar macrophages did not only self-maintain for months but retained an altered activation state that protected against the development of allergic asthma. Lung fibrosis was also associated with the differentiation of monocytes into self-maintaining alveolar macrophages with an altered gene-expression profile (Misharin et al., 2017). Whether the altered activation state of monocyte-derived alveolar macrophages in these models is imprinted locally by the virus infected or fibrotic lung or is in part determined by emergency monopoiesis in the BM is currently unknown. Moreover, whether these models of inflammation result in the production of a particular subset of monocytes possessing the unique capacity to self-maintain or to generate functionally distinct alveolar macrophages remains to be determined. Finally, it will be interesting to understand the epigenetic modifications of resident macrophages that were present prior to the inflammatory insult and that may also have undergone inflammatory training or tolerance. Is the long-term imprinting in the resident macrophages that were present before the inflammation comparable to the long-term imprinting in macrophages that derive from monocytes differentiating during the inflammation (Bonnardel and Guilliams, 2018)?

These examples demonstrate that monocytes can integrate into resident macrophage pools during inflammation, but whether these cells can self-maintain for prolonged periods appears to depend on the tissue and the inflammatory context. New fate mapping systems that faithfully label the monocyte-derived macrophages that engraft during inflammation will be required to assess their contribution to the local immune responses.

Conclusion and future directions

It is becoming clear that the substantial recruitment of monocytes to inflamed tissues does not merely function to temporarily increase the number of resident macrophages and DC. Instead, these infiltrating monocytes will often perform functions that cannot be performed by resident macrophages

or DC and will acquire very distinct functional profiles depending on the tissue and the inflammatory context. These monocytic phenotypes include effector monocytes with inflammatory, antigen presentation, regulatory or resident macrophage profile, although these phenotypes can overlap. Recently new monocyte subsets have been described under inflammatory as well as healthy conditions such as the neutrophil-like Ly6C^{Hi} monocytes, the SatM or the CD209^{+} monocytes. These cells have been proposed to display increased pro-inflammatory, pro-fibrotic and antigen-presentation capacities as compared to steady-state Ly6C^{Hi} monocytes. The identification of these cells highlights that single-cell analysis will profoundly reshape the monocyte field and may reveal the presence of monocytes primed for specific functions in many models. Future examination will be required to assess whether these monocytes indeed remain functionally distinct as compare to classical Ly6C^{Hi} that get recruited to the same inflamed tissue, since the local inflammatory cues may over-write the differences found in the circulating monocytes.

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Figures

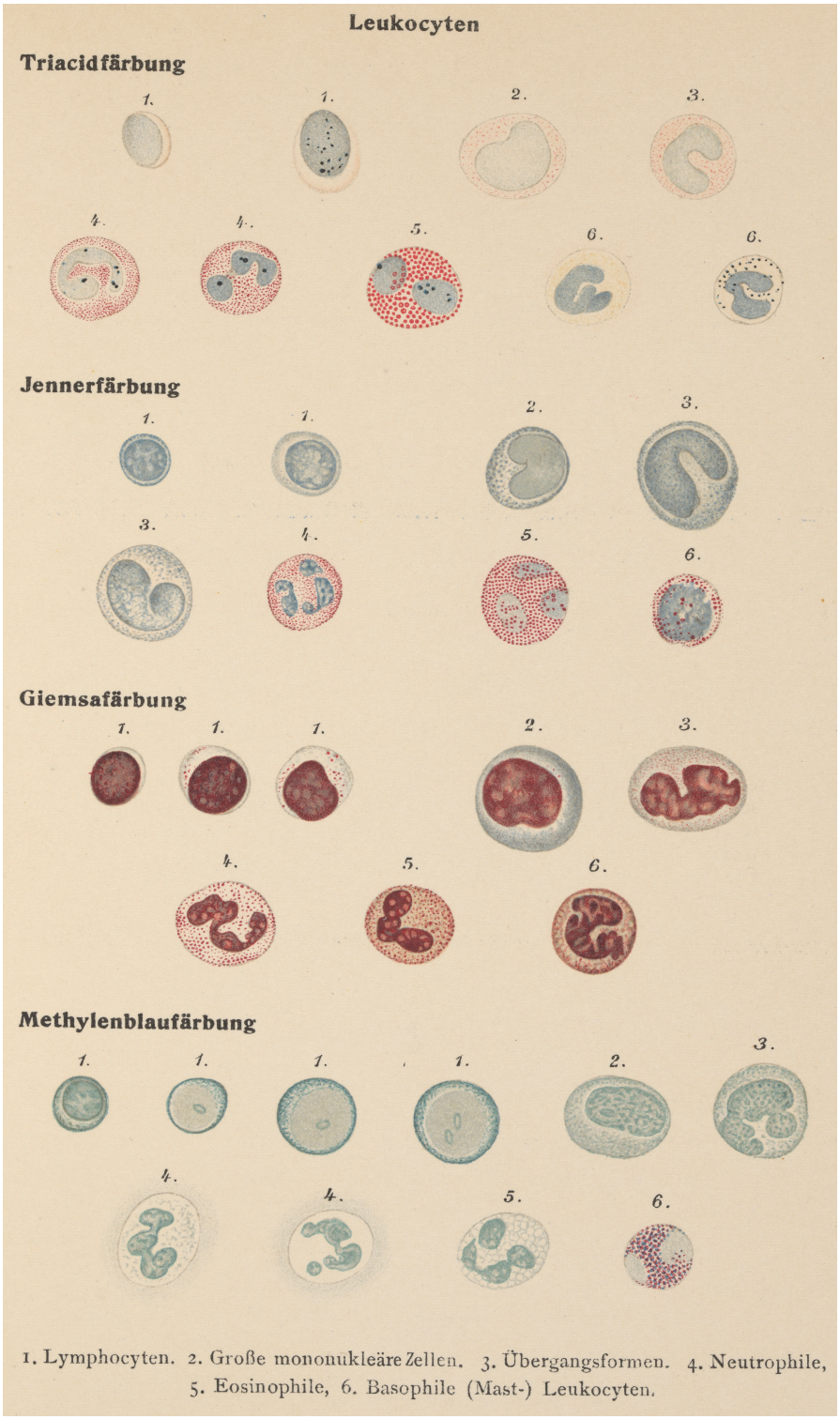

Figure 1: Historical leukocyte identification. By 1908, blood leukocytes could be stratified on the basis of their morphology and their staining properties. Different staining techniques are depicted here with 1. *Lymphocyten:* lymphocyte 2. *Grosse mononukleäre Zellen: Great* mononuclear cell 3. *Übergangsformen:* Ehrlich's transitional cell (monocytes) 4. *Neutrophile:* neutrophil 5. *Eosinophile: eosinophile* 6. *Basophile (Mast) Leukozyten:* basophil or mast cell (from (Naegeli, 1908)).

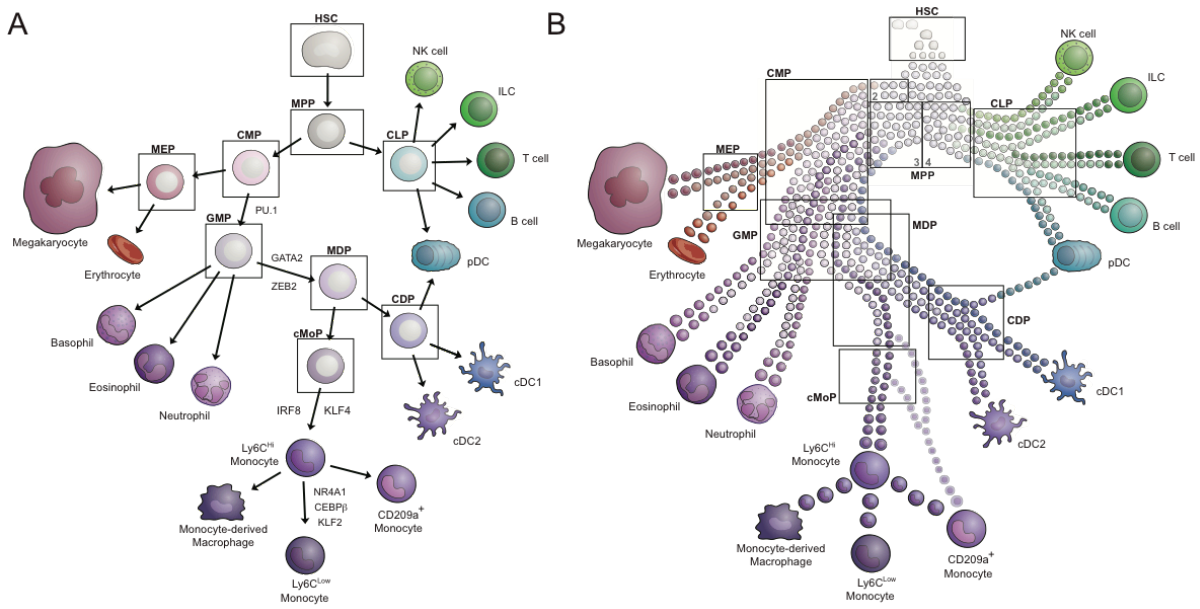

Figure. 2: Development of murine monocytes under physiological conditions. A. Depiction of the classic hierarchical representation of haematopoietic development in the bone marrow. Oligopotent precursors such as haematopoietic stem cells (HSC) or multipotent precursors (MPP) become progressively more restricted and give rise through intermediate precursor stages to all forms of circulating blood cells. In this view, defined multipotent precursor populations such as granulocyte macrophage precursors (GMP) produce either the granulocytic lineage or further differentiate into monocyte-macrophage/dendritic cell precursors (MDP). Bipotent MDPs further make binary decisions and either give rise to common dendritic cell precursors (CDP) or to common monocyte precursors (cMoP). cMoP finally differentiate into Ly6C^{Hi} cells, which are released into the circulation and can acquire different cell fates, including the transition into Ly6C^{Low} monocytes patrolling blood vessels or monocyte-derived macrophages in tissues. The main transcription factors involved in monocyte development are shown. CLP: common lymphocyte precursors. CMP: common myeloid precursors. MEP: megakaryocyte/erythrocyte progenitors. B. Development of the haematopoietic system based on single-cell analysis. In this view, haematopoietic development starts with few active HSC, which fill a pool of MPPs. Subsets of MPPs exist (indicated by the numbers 2-4 according to (Pietras et al., 2015)) that are already primed towards certain cell lineages or even show a cellular identity comparable (but not identical) to terminal differentiated cells (indicated by light coloured circles). Further differentiated precursor populations are therefore a heterogeneous mixture of primed cells (pool of cells in the rectangles in (B)), which share a – sometimes overlapping – common surface marker profile (rectangles corresponding to the supposedly homogeneous precursors shown in (A)).

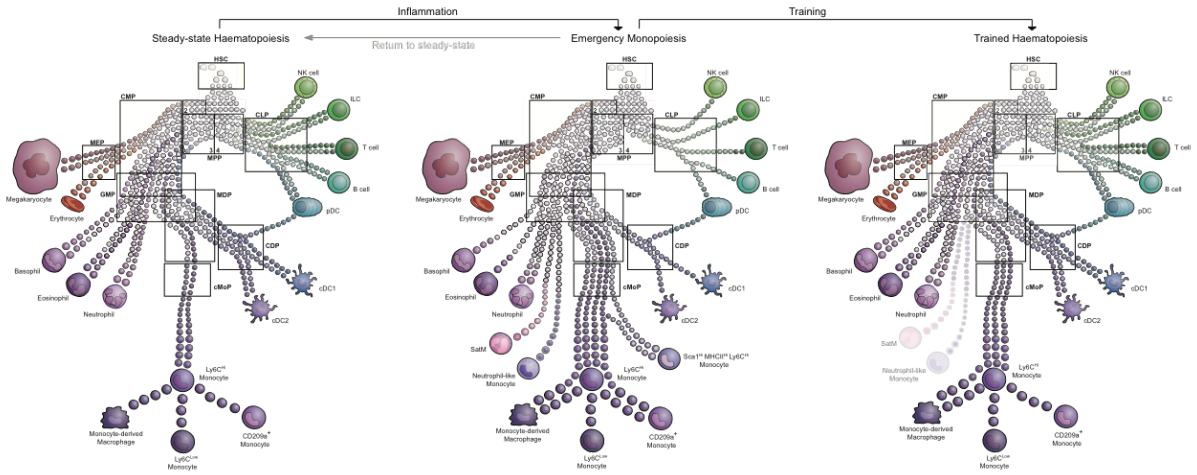

Figure. 3: Emergency monopoiesis and trained immunity. Compared to the physiological condition (left), severe inflammation can induce emergency monopoiesis leading to higher production of monocytes. Importantly, emergency monopoiesis also generates functionally distinct monocyte subsets with potentially alternative ancestry. These emergency-induced monocytes include neutrophil-like Ly6C^{Hi} monocytes and SatM that display neutrophil characteristics, but also Sca1^{Hi} monocytes with regulatory properties (middle). Emergency haematopoiesis can have long-lasting effects and yield in trained haematopoiesis characterized by a sustained increased production of monocytes that possess higher capacities to produce cytokines and superior microbicidal activities (right). The molecular mechanism that lead to the training effect and prevent haematopoiesis from returning to the steady-state after inflammation are starting to be revealed and involve IFN- and IL-1β-mediated signalling in HSCs.

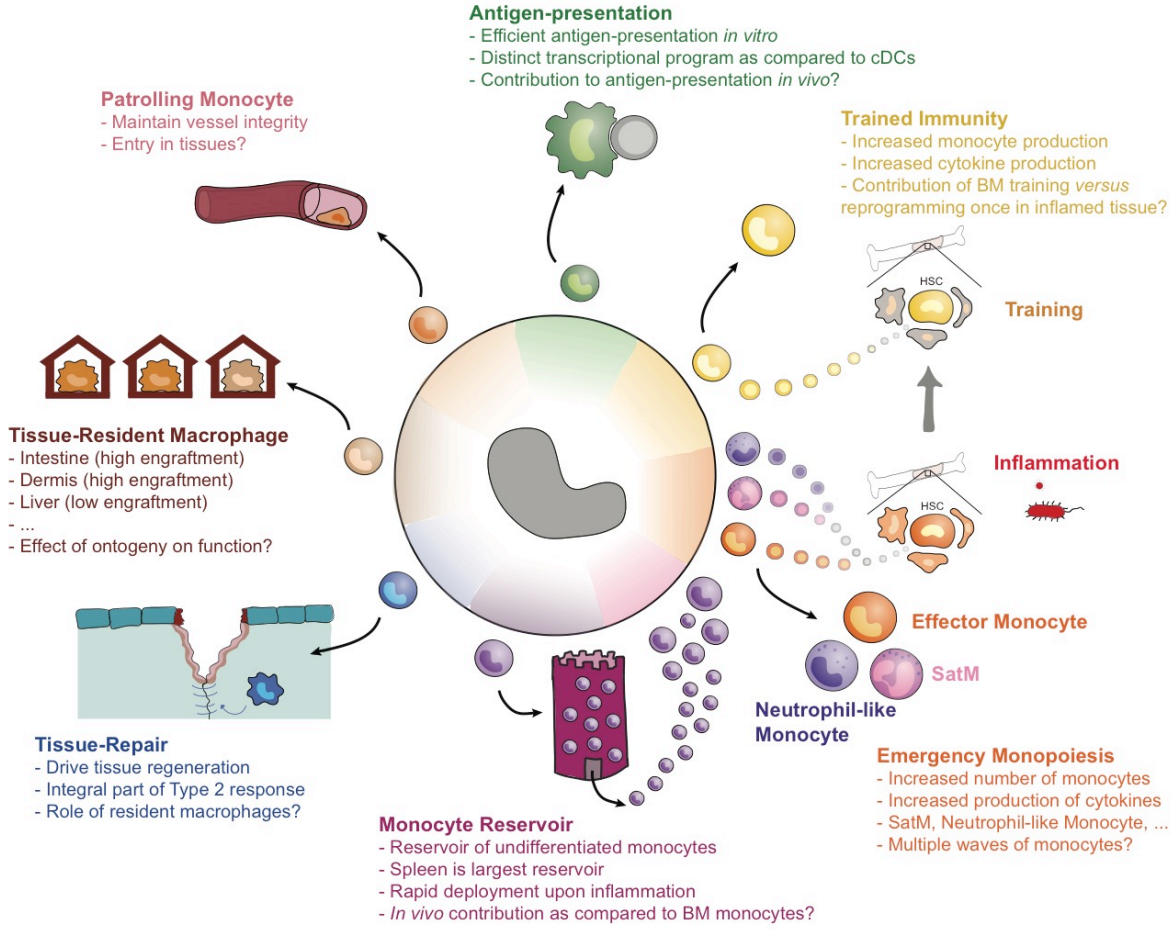

Figure 4: Diversity of monocyte functions during physiological and pathological conditions. Monocytes can engraft into peripheral monocyte reservoirs or can differentiate into non-classical patrolling monocytes or tissue-resident macrophages in the steady-state. Monocytes can also acquire inflammatory, regenerative or antigen-presentation capacities when entering inflamed tissues. Severe inflammation can also lead to emergency monopoiesis with the production of novel subsets of monocytes with altered functional characteristics. Inflammation can have long-term effects on haematopoiesis and result in trained immunity associated with increased output of monocytes producing more inflammatory cytokines.

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